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(54) Title: ENHANCED SECRETION OF HETEROLOGOUS PROTEINS BY HOSTS USING SUBSTITUTED PROMOTERS (57) Abstract <p>Improved secretion of heterologous proteins by hosts such as yeast by using promoters of at most intermediate strength with heterologous DNA secretion signal sequences. A promoter of at most intermediate strength, such as the actin (<i>ACT</i>) or iso-1-cytochrome <i>c</i> (<i>CYC1</i>) promoter in <i>S. cerevisiae</i> is operatively linked to a DNA signal sequence, such as the <i>MFa1</i> signal sequence. A DNA sequence for a selected protein, such as somatomedin C (SMC), tissue plasminogen activator (TPA) or tumor necrosis factor (TNF), may be operatively linked to the DNA signal sequence.</p>		

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ENHANCED SECRETION OF HETEROLOGOUS PROTEINS
BY HOSTS USING SUBSTITUTED PROMOTERS

Technical Field Of The Invention

5 This invention relates to expression systems
and recombinant DNA molecules that facilitate enhanced
secretion of heterologous proteins by hosts, to hosts
comprising such recombinant DNA molecules and to
methods of producing desired proteins using such
10 hosts.

Background Of The Invention

Proteins prepared by recombinant DNA methods
are sometimes difficult to isolate because the protein
must be extracted from the transformed microorganism
15 by means such as cell lysis that typically destroy
the microorganism. Obtaining sufficiently pure
protein in high yield from such extraction proce-
dures is difficult because of the large number of
different organic compounds liberated when lysis
20 takes place.

One attractive alternative to typical
separation procedures is to have the host secrete
the selected protein into its environment. A secreted
protein is more easily separated from the culture
25 medium, and the microorganism can survive the separa-
tion process to produce and to secrete more of the
desired protein.

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Most proteins naturally secreted by prokaryotes and eukaryotes are initially synthesized in the form of precursors containing an amino-terminal extension several amino acids long. This extension, called a secretion leader or signal sequence, allows the precursor protein to cross the cell membrane of the microorganism and enter the culture medium or periplasmic space of the cell. During secretion, the secretion leader is cleaved from the protein, leading to the presence of mature protein in the culture medium or periplasmic space.

Although the rate of secretion in bacteria is typically very low, bacterial secretion has been used with recombinant DNA technology. See, for example, United States Patents 4,411,994 and 4,338,397, and Villa-Komaroff et al., Proc. Natl. Acad. Sci. USA, 75, 3727-31 (1978).

The general safety of yeasts and human experience with yeast fermentation have made yeasts desirable candidates for use as hosts in recombinant DNA technology. However, reported attempts to obtain secreted mature proteins from recombinant yeasts have suffered from low yields. See, for example, C. N. Chang et al., "Recognition and Cleavage of Hybrid Invertase Signals and Mature Forms of Human Interferon (IFN- α 2) in Yeast," Meeting Abstracts, The Molecular Biology of Yeast, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p. 393 (1983); B. Meyhack and A. Hinnen, "High Levels of Expression of Foreign Genes Under the Control of the Yeast PHO5 Promoter," Meeting Abstracts, The Molecular Biology of Yeast, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p. 156 (1983); S. D. Emr, "An MF α 1-SUC2 (α -Factor-Invertase) Gene Fusion for Study of Protein Localization and Gene Expression in Yeast," Proc. Natl. Acad. Sci. USA, 80, pp. 7080-84 (1983); A. Brake et al., " α -Factor-Directed Synthesis

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and Secretion of Mature Foreign Proteins in Saccharomyces cerevisiae," Proc. Natl. Acad. Sci. USA, 81, pp. 4642-46 (1984); G. A. Bitter et al., "Secretion of Foreign Proteins from Saccharomyces cerevisiae Directed by α -Factor Gene Fusions," Proc. Natl. Acad. Sci. USA, 81, pp. 5330-34 (1984); A. Singh, et al., "Synthesis, Secretion and Processing of α -Factor-Interferon Fusion Proteins in Yeast," Nucl. Ac. Res., 12, pp. 8927-38 (1984); European Patent Application 123,228; and European Patent Application 128,733; and G. Bitter et al, "Secretion of Foreign Proteins from Saccharomyces cerevisiae Directed by α -Factor Pheromone," Nucl. Ac. Res., 11, pp. 4049-63 (1983).

15 Summary of the Invention

The present invention solves the foregoing problems by providing expression systems that facilitate enhanced secretion of heterologous proteins. We have found that using a promoter of at most intermediate strength and a heterologous DNA secretion signal sequence in a multicopy vector results in a higher yield of a desired protein from the host compared to hosts transformed by a multicopy vector using a strong promoter.

25 Thus, one embodiment of the present invention comprises a DNA sequence that is an expression and secretion control sequence for producing and secreting a selected protein from a host in which the protein is made, said sequence comprising (a) a promoter that is active in said host and is of at most intermediate strength, and (b) a heterologous DNA secretion signal sequence, recognized by said host, beginning with a start codon, operatively linked to said promoter. The expression and secretion control sequence may then be operatively linked to a DNA sequence coding for a desired protein and employed

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to transform a host to produce and secrete the desired protein. Yeast is a preferred host, and the actin (ACT) and iso-1-cytochrome c (CYC1) promoters are preferred promoters for yeast hosts. S. cerevisiae is the most preferred host. When the host is S. cerevisiae, the secretion signal sequence is preferably the secretion signal sequence of the MF α 1 gene.

The present invention also relates to hosts transformed by the recombinant DNA molecules discussed above and to methods of using transformed hosts in the preparation and secretion of desired proteins.

Brief Description Of The Drawings

Figure 1A shows the structure of the MF α 1 secretion precursor. Stippled regions indicate spacer regions separating the MF α 1 repeats. During secretion the secretion leader is cleaved off at the position indicated by the arrow.

Figure 1B shows a general scheme for the construction of a yeast secretion precursor comprising the alpha mating factor (MF α 1) secretion leader fused to the desired heterologous protein. During secretion, the secretion leader is cleaved at the position indicated by the arrow.

Figure 2 shows the construction of a gene fusion between the MF α 1 expression control sequence and the SMC gene and insertion of the MF α 1/SMC fusion into a yeast expression vector.

Figure 3 shows two graphs: growth (as reflected by optical density at 600 nm) and SMC levels in the culture fluid of yeast strain BJ1991 transformed with URA3-type expression vectors. The CYC1 promoter construction (p336/1) is shown by (●); the ACT promoter construction (p364/1) by (▲); and the MF α 1 promoter construction (p446/1) by (■).

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Figure 4A shows the construction of actin promoter fragments ending in EcoRI sites.

Figure 4B shows the DNA structure of promoter ends for actin and the pEX-5, pEX-7, and pEX-8 vectors.

Figure 5 shows the construction of SMC secretion vectors based on the ACT and CYC1 promoters.

Figure 6 shows the structure of SMC secretion vectors based on the yeast LEU2 gene.

Figure 7 shows two graphs: growth (as reflected by optical density at 600 nm) and SMC levels in the culture fluid of yeast strain BJ1991 transformed with LEU2-type expression vectors. The CYC1 promoter construction (504/1) is shown by (●); the ACT promoter construction (p482/18) by (▲); and the MEal promoter construction (pMF-SMC, rearranged) by (■).

Figure 8 shows the construction of secretion vectors for TNF.

Figure 9 shows the construction of secretion vectors for TPA.

Detailed Description Of The Invention

In order that the invention may be more fully understood, the following detailed description is provided. In this specification, some of the following terms are used:

Cloning -- the process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Recombinant DNA Molecule or Hybrid DNA -- A molecule consisting of sequences of DNA from different genomes which have been joined end-to-end outside of living cells and that can be maintained in living cells.

Protein -- A polypeptide containing a linear series of more than fifty amino acids, e.g., pro-

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insulin, serum albumin, human growth hormone, parathyroid hormone, and interferon. As used herein, however, a protein also comprises a polypeptide chain of fewer than fifty amino acids.

5 Polypeptide -- A linear series of amino acids connected one to the other by peptide bonds between the amino and carboxy groups of adjacent amino acids.

10 Expression -- The process undergone by a gene to produce a polypeptide or protein. It is a combination of transcription and translation.

Transcription -- The process of producing mRNA from a gene.

15 Translation -- The process of producing a protein or polypeptide from mRNA.

Promoter -- The region of DNA responsible for binding RNA polymerase to initiate transcription. In bacterial expression systems a promoter is located before the ribosome binding site. A strong promoter
20 is defined as one that has a strong affinity for RNA polymerase and that would accordingly be expected to aid in obtaining high expression rates. A promoter of intermediate strength has a lower affinity for RNA polymerase.

25 In recombinant systems, the strength of a promoter may be affected by the host, the desired protein and the expression vector of the recombinant system. The effects of these factors are more fully discussed below. In systems that do not secrete the
30 desired protein, i.e., intracellular expression systems, levels of mRNA and levels of expressed protein are proportional to promoter strength. In intracellular expression systems, no changes of plasmid copy number have been observed with variation
35 of promoter strength. J. Mellor et al, "Factors Affecting Heterologous Gene Expression in Saccharomyces cerevisiae," Gene, 33, pp. 215-26

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(1985). In secretion expression systems, we have discovered that promoter strength may affect the number of copies of a multicopy expression vector produced within a given host. In secretion expression systems, a host will produce many copies of a multicopy vector that contains an intermediate or weak promoter but will produce fewer copies of a multicopy vector that contains a strong promoter. An estimate of the strength of a promoter may be based on the percent of total mRNA in a cell produced by the expression control sequences of that promoter. Table 1 shows a list of genes, including their associated promoters and associated mRNA, as described in the literature. A characterization of the promoter strength is also included.

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TABLE 1

	Gene	Protein	Percent of Total mRNA	Reference	Character- ization of Promoter Strength
5	pgap 63	G3PDH	1.5-6	1	very strong
	peno 8	enolase	1-3	1	very strong
	ADH-I	alcoholde- hydrogenase	0.7-2	1	very strong
10		histone 2B	0.4	1	strong
	tcm	ribosomal protein P1	0.3	2, 3	strong
	act	actin	0.15	3	intermediate
	cycl	iso-1- cytochrome <u>c</u>	0.05	1	weak
15	URA3	-	0.008	2	very weak
	cyc7	iso-2- cytochrome <u>c</u>	0.003	1	very weak
	trp1	-	<0.01	4	very weak

20

1. J. L. Bennetzen and B. D. Hall, "Codon Selection in Yeast," J. Biol. Chem., 257, pp. 3026-31 (1982).
2. C. H. Kim and J. R. Warner, "Messenger RNA for Ribosomal Proteins in Yeast," J. Mol. Biol., 165, pp. 78-89 (1983).
3. H. J. Himmelfarb et al., "Isolation of the SUP45 Omnipotent Suppressor Gene of Saccharomyces cerevisiae and Characterization of its Gene Product," Mol. Cell. Biol., 5, pp. 816-22 (1985).
4. M. J. Dobson et al., "Expression in Saccharomyces cerevisiae of Human Interferon-Alpha Directed by TRP1 5' Region," Nucl. Ac. Res., 11, pp. 2287-2302 (1983).

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Generally, promoters of glycolytic genes are considered "strong." Those skilled in the art will understand, however, that promoters may not have the same strength within different strains of a host, with different desired proteins or in different multicopy vectors. In S. cerevisiae, for example, the ME_α1 promoter is active in MAT_α strains, but inactive in MAT_a strains. Those skilled in the art will appreciate that promoter strength depends upon many factors. Merely because a promoter is unacceptable with respect to one combination of host, vector and desired protein does not mean that the promoter will not be acceptable for another combination of host, vector and desired protein.

Promoters of the present invention should be selected so that the combination of promoter strength and copy number of the multicopy vector provides optimum yields of desired protein. Promoters of intermediate strength provide the best combination of promoter strength with copy number. Weaker promoters, in combination with high copy numbers, are also within the scope of the invention. Strong promoters adversely affect the copy number of the multicopy vectors within the transformed host, and unacceptable yields are obtained from those transformants. A promoter should be selected that has maximal strength without significantly reducing plasmid copy number. Generally, a promoter may be considered of at most intermediate strength with respect to a given combination of host, vector and desired protein if the percent of total mRNA in the host corresponding to the promoter is less than 0.3%, and preferably is at most 0.15%.

Ribosome Binding Site -- The region of DNA which codes for a site on mRNA which helps the mRNA bind to the ribosome, so that translation can begin. In bacterial expression systems, a ribosome binding

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site is located after (downstream from) the promoter and before (upstream from) the translational start signal of the DNA sequence to be expressed to produce the desired protein.

5 Gene -- A DNA sequence which encodes, as a template for mRNA, a sequence of amino acids characteristic of a specific polypeptide or protein.

Expression Control Sequence -- A DNA
10 sequence that controls and regulates expression of genes when operatively linked to those genes. Such sequences include the lac system, the β -lactamase system, the trp system, the tac, and trc systems, the major operator and promoter regions of phage λ , the control region of fd coat protein, the early and
15 late promoters of SV40, promoters derived from polyoma virus and adenovirus, metallothionine promoters, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating
20 factors, and other sequences known in the art to control the expression of genes in prokaryotic or eukaryotic cells and their viruses or combinations thereof. For mammalian cells, the gene can be linked to a eukaryotic promoter, such as that for the SV40
25 early region, coupled to the gene encoding dihydrofolate reductase and selectively amplified in Chinese hamster ovary cells to produce a cell line containing many copies of actively transcribed eukaryotic genes. Those skilled in the art will appreciate
30 that not every expression control sequence listed above as an example may be suitable for use with each host, desired protein and vectors combination.

Precursor of a Protein -- A protein with a signal sequence operatively linked to the protein.
35 Typically, the precursor is synthesized within a host cell, e.g., preproinsulin, prealbumin, prehuman growth hormone, parathyroid hormone, and preinter-

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feron. In accordance with this invention, a mature protein is obtained by secreting the precursor through the cell membrane of a host with an attendant loss or clipping of the signal sequence of its precursor.

5 Nucleotide -- A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C") and thymine ("T"). The four RNA bases are A, G, C and uracil ("U").

10 DNA Sequence -- A linear series of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon -- A DNA sequence of three nucleotides (a triplet) which encodes, through messenger RNA ("mRNA"), an amino acid, a translational start signal or a translational termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translational stop signals and ATG is a translational start signal.

20 Plasmid -- A non-chromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a host organism, the characteristics of that organism are changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet^R) transforms a host cell previously sensitive to tetracycline into one which is resistant to it. A host cell transformed by a plasmid is called a "transformed host" or a "transformant."

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Phage or Bacteriophage -- Bacterial virus which may include DNA sequences contained in a protein envelope or coat ("capsid").

Cloning Vehicle -- A plasmid, phage DNA or
5 other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at which its DNA sequence may be cut in a determinable fashion without attendant loss of an essential biological function
10 of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites, and which contains a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A cloning
15 vehicle is also known as a vector. In the present invention, the vector must be a "multicopy" vector, i.e., capable of producing multiple copies of itself within the host. Single copy vectors, incapable of producing copies, are not within the scope of this
20 invention.

Host -- An organism which on transformation by a cloning vehicle enables the cloning vehicle to replicate and to accomplish its other biological functions, e.g., the production of polypeptides or
25 proteins through expression of the genes of a plasmid.

DNA Signal Sequence -- A DNA sequence which encodes, as a template for mRNA, a sequence of typically hydrophobic amino acids at the amino terminus of the polypeptide or protein, i.e., a
30 "signal sequence" or "secretion leader sequence" of the protein. For secretion, such a DNA signal sequence is operatively linked to and located immediately before the DNA sequence of the desired protein and after its translational start signal (e.g., ATG).
35 It is believed that only a portion of a signal sequence of a precursor of a protein is essential for the precursor of the protein to be transported

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through the cell membrane of a host and for proper clipping of the precursor's signal sequence to free the protein during secretion. Combinations of signal sequences or parts of signal sequences may also be used provided that proper processing takes place in the host. Hence, the term "DNA signal sequence" as used herein includes those DNA sequences that code for that portion of a signal sequence required for secretion.

10 In accordance with this invention, to obtain expression of a desired protein, an expression system of this invention is operatively linked to a DNA sequence coding for the desired protein and used to transform an appropriate host. The host may then be
15 cultured under appropriate conditions of growth. The desired protein may then be isolated from the culture. Optimum results will, of course, depend on a number of variables, including the appropriate choice of host, promoter, vector, and growth conditions.
20

Many hosts have been used in recombinant DNA technology and are well known in the art. A wide variety of hosts may be useful in the method of this invention. These hosts include for example,
25 bacteria, such as E.coli (for example E.coli HB101 or E.coli MC1061), Bacillus, Streptomyces, and Pseudomonas, fungi, such as yeasts, animal cells, such as CHO cells, mouse, swine, bovine, fowl or fish cells, plant cells in tissue culture, human
30 tissue cells, or other hosts known in the art.

The selection of an appropriate host is controlled by a number of factors recognized by the art. These include, for example, compatibility with the chosen vector, ease of recovery of the desired
35 protein, expression characteristics, biosafety and costs. In the present invention, the host must be able to recognize both the promoter used and

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the secretion signal sequence used. In addition, the toxicity of the desired protein on the host must be considered, as well as the viability of the host when transformed by a multicopy vector. No absolute
5 choice of host may be made for a particular recombinant DNA molecule or polypeptide from any of these factors alone. Instead, a balance of these factors must be struck with the realization that not all hosts may be equally effective for expression of a
10 particular DNA sequence operatively linked to a particular expression control sequence. While the present invention is not limited to S. cerevisiae, yeast is a preferred host, and S. cerevisiae is especially preferred.

15 Various promoters may be used in the expression systems of this invention, provided, however, that the promoter is active in the chosen host and is of at most intermediate strength.

The toxicity of the desired protein to the
20 host affects the promoter choice; for secretion of a less toxic protein, a relatively stronger promoter can be used than for secretion of a more toxic protein. The coding region attached to a promoter in a multicopy vector also affects the strength of the
25 promoter. For example, the pgk promoter is very strong when attached to its own coding region (PGK), but weak when attached to an alpha interferon gene. J. Mellor et al., "Factors Affecting Heterologous Gene Expression in Saccharomyces cerevisiae," Gene, 33,
30 pp. 215-26 (1985). In choosing a promoter it should also be appreciated that the junction of a strong promoter to a heterologous gene will considerably weaken the strong promoter, as described by Mellor et al., supra. Because promoter strength is affected
35 by so many variables, the fact that a promoter is too strong for use in one host-vector-desired protein

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combination does not exclude the use of that promoter in a different combination.

Promoters that may be considered for use in recombinant DNA molecules made from the present invention include, for the yeast S. cerevisiae, the promoters of the ACT (actin) gene, the CYC1 (iso-1-cytochrome c) gene and the URA3 gene. Kim and Warner, J. Mol. Biol., 165, pp. 79-89 (1983) (mRNA level about 0.008%). For secretion expression systems, strong promoters not suitable for use with S. cerevisiae include, for example, the G3PDH and the enolase promoters.

Preferred promoters in secretion expression systems for use with bacteria such as E.coli and B. subtilis should be of at most intermediate strength. Strong promoters, such as the trp, trc, lac promoters should generally be avoided. Intermediate promoter strength might, however, conveniently be adjusted with regulated promoters, such as the trp or lac promoters, if intermediate inducing growth conditions are chosen.

In the examples, we have used promoters that are active during all phases of growth. If the promoters were to be shut off during growth, the adjustment of vector copy number would presumably not take place unless the promoters were induced. Regulated promoters, however, would probably not provide an advantage. Presumably, turning on a strong promoter in a high-copy secretion construction would immediately lyse the host, as in insulin secretion by E.coli.

As discussed above, we have discovered that, for secretion expression systems, a host makes few copies (i.e., low copy numbers) of multicopy vectors if the promoter is strong in the host-vector-desired protein system, and more copies (i.e., higher copy numbers) are made of multicopy vector having a weaker

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promoter, resulting in improved yields of desired protein. However, the use of a promoter weaker than the CYC1 promoter discussed in Example 3 (e.g., CYC7 or trp1) will not improve yields due to higher copy numbers, because maximal copy numbers are seen for the CYC1 promoter construction (Table 2). Lowest strength promoters are limited by the promoter strength, and the strongest promoters give only limited copy numbers. Though not wishing to be bound by any theory, it appears that high copy numbers combined with strong promoters result in high levels of mRNA, oversecretion of the desired protein or both. In a population of transformants containing cells transformed by vectors having strong promoters, a mixture of copy numbers occurs in the population. Those cells having high copy numbers with strong promoters will stop growing and eventually lyse, while those transformants with lower copy numbers and the same strong promoter will survive. The population will eventually contain only low copy number transformation. See generally T. J. Silhavy, et al., "Mechanisms of Protein Localization," Microbiol. Res., 47, pp. 313-34 (1983).

The multicopy vector and, in particular, the sites chosen therein for insertion of the expression systems of this invention are determined by a variety of factors, e.g., number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, expression characteristics such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector is determined by a balance of these factors, not all selections being equally effective for a given case. The preferred vectors are plasmids, such as those containing origins of replication derived from chromosomal and non-chromosomal DNA. In the yeast

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S. cerevisiae, the various ARS- and 2 μ -type vectors (Botstein et al.) are preferred. In E.coli, multicopy plasmids, such as ColE1-, pBR322 and RP4 plasmids, or M13 and lambda phage vectors, can be used. For
5 animal cells, non-integrating multicopy vectors, such as vectors based on the bovine papilloma virus, are preferred. The choice of vector will also be influenced by the chosen promoter. optimal promoter and vector systems will lead to less toxicity of the
10 secreted protein (due to too high copy number or over production based on too strong a promoter) and consequently, higher levels of secretion may be obtained.

The heterologous secretion signal sequences
15 of this invention must be recognized and correctly processed by the host. The signal sequence may comprise a naturally occurring signal sequence, an effective portion of a naturally occurring signal sequence, or a combination of two or more signal sequences or
20 effective portions of signal sequences. The signal sequence must be operatively linked to both the promoter and the start signal, ATG. Preferably, the signal sequence begins with the translation start signal. In a preferred embodiment, the signal
25 sequences is selected from signal sequences already used by the host to aid secretion of its own proteins.

The desired protein may include any polypeptide or protein, and may include fusions, preproteins, immature proteins or any desired sequence of
30 amino acids. Preferably, however, desired proteins of this invention include proteins, and other amino acid sequences, that are secretable by some micro-organism or cell. Examples of such secretable
35 proteins and other amino acid sequences include serum proteins, analgesic polypeptides such β -endorphin, somatostatin, insulin, growth hormone

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(human and bovine), luteinizing hormone, ACTH, pancreatic polypeptide preproteins, preproinsulin, proinsulin, and the A and B chains of insulin. The desired proteins of the present invention may comprise
5 proteins and other amino acid sequences naturally secreted into the medium by the selected host.

The desired protein of the present invention need not be secreted by the selected host. As will be seen in Example 6, the host need only manufacture the desired protein operatively linked to
10 the chosen signal sequence and correctly process the desired protein into the endoplasmic reticulum. If the desired protein enters the secretion pathway of the host, benefits may be obtained for the desired
15 protein, such as glycosylation. Secretion is, however, the preferred result for the desired protein.

The present invention may be useful in mammalian cells, if non-integrating, multicopy vectors, such as vectors derived from bovine
20 papilloma virus (D. DiMaio et al., "Bovine Papilloma-Virus Vector that Propagates as a Plasmid in Both Mouse and Bacterial Cells," Proc. Nat. Acad. Sci. USA, 79, pp. 30-34 (1982)) are used in conjunction with an intermediate strength promoter.

25 The following, non-limiting examples will serve to further illustrate the present invention.

Example 1

Preparation Of MF α 1/SMC DNA Sequences

This example describes the preparation of
30 a transformed yeast which is used in a later example for comparative purposes. The transformed yeast of this example has a plasmid containing a MF α 1 promoter (which is a strong promoter in yeast as shown below) operatively linked to a MF α 1 secretion leader and a
35 DNA sequence coding for SMC. Other examples describe

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the substitution of the ACT and CYC1 promoters for the MF α 1 promoter, and a comparison of the secretion levels of the variously transformed yeasts. In addition, other examples describe the preparation of transformed yeasts having plasmids characterized by DNA sequences coding for desired proteins other than SMC.

We first isolated the gene for pre-MF α 1 and its associated expression control systems and we then operatively linked the MF α 1 promoter and signal sequence to a DNA sequence for SMC.

The DNA sequence coding for the precursor of MF α 1 has been sequenced by J. Kurjan and I. Herskowitz, "Structure of a Yeast Pheromone Gene (MF α): A Putative α -Factor Precursor Contains Four Tandem Copies of Mature α -Factor," Cell, 30, pp. 933-43 (1982). We isolated the gene coding for those pre-MF α 1 and its associated expression control sequences from S. cerevisiae using the library constructed by K. A. Nasmyth, and S. I. Reed, "Isolation of Genes by Complementation in Yeast: Molecular Cloning of a Cell-Cycle Gene," Proc. Natl. Acad. Sci. USA, 77, 2119-23 (1980). Instead of using the cdc28 mutant described in Nasmyth and Reed for isolation and cloning of the cdc28 gene, we used the following oligonucleotide corresponding to amino acids 97 to 102 of the published pre-MF α 1:

(5') GTA CAT TGG TTG C/GCC G/A/TGG (3')

The MF α 1 secretion precursor, comprising a secretion leader and four MF α 1 repeats is shown in Figure 1A. The stippled regions indicate spacer regions between the MF α 1 repeats. Before substituting a desired heterologous protein for the four MF α 1 repeats, as shown in Figure 1B, the pre-MF α 1 gene and associated expression control sequence, which resided on a 1.7 kb EcoRI fragment, were subcloned into pUC18, the selected cloning vector. The result-

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ing plasmid (p220/3) now included an a MF α 1 promoter, an MF α 1 signal sequence and an MF α 1 DNA coding sequence. In order to isolate the MF α 1 coding sequence from the MF α 1 promoter and MF α 1 signal sequence, we subjected plasmid p220/3 to mutagenesis using the procedure described in B. A. Oostra et al., "Transforming Activity of Polyoma Virus Middle-T Antigen Probed by Site-Directed Mutagenesis," Nature, 304, pp. 456-59 (1983). As a result of the mutagenesis, a convenient BglIII site was introduced at the junction between the secretion leader and the coding sequence for the repeats in the MF α 1 DNA sequence as follows:

modified
 15 oligomer: (3') TA TTT TCT CTA GAA CTT CGA ACC (5')
 original: (3') TA TTT TCT CTC CGA CTT CGA ACC (5')
 sequence lys arg glu ala glu ala trp

The resulting plasmid was called p254 (Figure 2). It was further modified to prevent unintended cleavage at a BglIII site found at the 5' end of the MF α 1 promoter. We altered this undesired BglIII site by cleaving with BglIII and filling in the protruding ends using the large (Klenow) fragment of DNA polymerase I and deoxynucleotide triphosphates and subsequent ligation using T4 ligase. To allow for a different cleavage in the plasmid (Examples 5 and 6), a StuI site was introduced, in a procedure similar to the mutagenesis described above, at the position corresponding to the same lys-arg cleavage site:

modified
 oligomer: (5') TA AAA AGG CCT CTT GAA GC (3')
BglIII-sequence: (5') TA AAA AGA GAT CTT GAA GC (3')
 lys arg

35 We then made a MF α 1/SMC (secretion leader/desired protein) fusion by inserting a 500 bp HindIII fragment carrying a synthetic SMC gene starting with

-21-

a unique NcoI site into the HindIII site of plasmid p254 (described above) which contained the MF α 1 promoter and secretion leader and obtained plasmid F-9 as shown in Figure 2. The synthetic SMC gene is described in G. Buell et al., "Optimizing the Expression in E.coli of a Synthetic Gene Encoding Somatomedin-C (IGF-1)," Nucl. Ac. Res., 13, pp. 1923-38 (1985). We then cut plasmid F-9 with BglII and NcoI, with simultaneous S1 treatment and religation to remove the Δ portion of plasmid F-9 and obtain pS30/25, shown in Figure 2. The correct fusion of the MF α 1 secretion leader and the SMC structural DNA sequence had the following sequence (glycine is the first amino acid of SMC):

(secretion leader)-AAA-AGA-GGT-CCA-(SMC)
lys arg gly pro

As a result the SMC DNA sequence was operatively linked to the MF α 1 secretion leader so that the SMC protein would be properly secreted by yeast.

As outlined in Figure 2, we introduced the MF α 1/SMC fusion into a yeast shuttle vector carrying origins of replication for E.coli and yeast (ori and the 2 μ origin of replication, respectively), as well as selectable markers for both organisms (E.coli: bla; yeast: URA3). SMC secretion rates by S. cerevisiae transformed with this expression vector are shown in Figure 3.

Example 2

Preparation Of ACT/MF α 1/SMC DNA sequences

The DNA sequence encoding actin in yeast is described in D. Gallwitz and I. Sures, "Structure of a Split Yeast Gene: Complete Nucleotide Sequence of the Actin Gene in Saccharomyces cerevisiae," Proc. Natl. Acad. Sci. USA, 77, pp. 2546-50 (1980). Levels of the mRNA coding for actin (D. Gallwitz et al.,

-22-

"The Actin Gene in the Yeast Saccharomyces cerevisiae: 5' and 3' End Mapping, Flanking and Putative Regulatory Sequences," Nucl. Ac. Res., 9, pp. 6339-50 (1981)) suggest that the ACT promoter is a promoter of intermediate strength, see Table 1. See also Himmelfarb, supra.

In order to use the ACT promoter in the constructions of this and the following examples, we placed convenient restriction sites at the end of the promoter. Plasmid pYA301, shown in Figure 4, contains the ACT gene and its associated expression control sequences on a 4 kb EcoRI-BamHI fragment. Plasmid pYA301 is a 4 kb EcoRI-BamHI subclone of pYA208 (Gallwitz and Sures, supra) inserted into pBR322. We cut plasmid pYA301 at the single XhoI site and treated it with Bal31. Following an empirically determined time of Bal31 treatment, we cut the plasmid with EcoRI and its protruding ends were filled in using the large (Klenow) fragment of DNA polymerase I. Finally, the treated plasmid was religated under dilute conditions. Approximately half of the resulting plasmids, identified collectively as pA4, contained an EcoRI site. These EcoRI sites had been formed by joining the filled-in EcoRI end to the Bal31 ACT promoter end. Structures of various pA4 promoter ends in the vicinity of the ATG start codon of the religated plasmid are shown in Figure 4B.

To operatively link the isolated ACT promoter to the MEal signal sequence SMC fusions, we matched the 3' end of the ACT promoter to the amino terminal end of the MEal/SMC fusion. We accomplished this by introducing an EcoRI site upstream (i.e., earlier in the transcription reading direction) of the ATG start codon of the MEal secretion leader by carrying out the following mutagenesis using the procedure described in Oostra et al., supra:

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modified
oligomer: (5') A ATA TAA ACG AAT TCA AGA ATG AG (3')

original
sequence: (5') A ATA TAA ACG ACC AAA AGA ATG AG (3')

5 We named the resulting plasmid p269/20.

We joined the BamHI-EcoRI fragment of pEX-7 (Fig. 4) isolated above and containing the ACT promoter to the EcoRI-SalI fragment of plasmid p269/20 (which carried the MF α 1 secretion leader) and inserted the fragment into a BamHI and SalI cut pUC8 vector as shown in Figure 5. We further modified the resulting plasmid (p309/1) by replacing the MF α 1 secretion leader region of plasmid p309/1 by the secretion leader region, joined to the SMC gene, of plasmid pS30/25. Plasmid pS30/25 is shown in Figure 2. The modification was carried out by cutting plasmid p309/1 with PstI and HindIII. The large fragment was isolated and joined to a fragment of pS30/25 resulting from partial digestion of that plasmid with PstI and HindIII. The resulting plasmid, p355/1, (ACT promoter/MF α 1 secretion leader/SMC structural DNA sequence) was transferred to a yeast shuttle vector, as shown in Figure 5, which resulted in formation of plasmid p364/1. SMC secretion rates by yeast transformed with this plasmid are shown in Figure 3.

EXAMPLE 3

Preparation Of CYC1/MF α 1/SMC Plasmids

Under inducing conditions, iso-1-cytochrome c (CYC1) amounts to approximately 0.2% of the total cellular protein of yeast, and the encoding mRNA amounts to about 0.05% of the total poly(A) RNA. R. S. Zitomer and B. D. Hall, "Yeast Cytochrome c Messenger RNA. In Vitro Translation and Specific Immunoprecipitation of the CYC1 Gene Product,"

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J. Biol. Chem., 251, 6320-26 (1976). The CYC1 promoter is thus a weak promoter.

In order to construct a CYC1 promoter/MF α 1 secretion leader/SMC coding sequence, we isolated an
5 EcoRI-HindIII fragment carrying the MF α 1 secretion leader correctly fused to the SMC gene from plasmid p355/1, which is shown in Figure 5. We inserted this fragment between the EcoRI and HindIII sites of pEX-2 to obtain plasmid p446/1 (See Figure 5).
10 pEX-2 is described in J. F. Ernst and R. C. Chan, "Characterization of S. cerevisiae Mutants Supersensitive to Aminoglycoside Antibiotics," J. Bacteriol., 163, pp. 8-14 (1985).

The vector p446/1 expressed the MF α 1/SMC
15 fusion under the control of the CYC1 promoter. SMC secretion rates from yeast transformed by this vector are shown in Figure 3.

• EXAMPLE 4

20 Comparison Of Secretion Of SMC Using The MF α 1, ACT And CYC1 Promoters With Both The URA3 Gene And The LEU2 Gene on Expression Vectors

The construction of tripartite SMC expression vectors based on the MF α 1 secretion leader and the MF α 1, the ACT, and the CYC1 promoters has been
25 described above. Each of these vectors contained the yeast URA3 gene for selection in yeast. We also constructed vectors based on selection using the yeast LEU2 gene. The expression of the plasmid LEU2 gene is low due to a partial deletion; higher copy numbers
30 of the LEU2 type expression vectors are needed to allow the growth of transformants on leucine-free media. E. Erhart and C. P. Hollenberg, "The Presence of a Defective LEU2 Gene on 2 μ DNA Recombinant Plasmids of Saccharomyces cerevisiae is Responsible
35 for Curing and High Copy Number," J. Bacteriol., 156, pp. 625-35 (1983). For a discussion of the use

-25-

of yeasts having the URA3 gene or the LEU2 gene in recombinant DNA experiments, see D. Botstein et al., "Sterile Host Yeast (SHY): a Eukaryotic System for Biological Containment for Recombinant DNA Experiments," Gene, 8, pp. 17-24 (1979).

We constructed the LEU2-type vectors by inserting the chosen promoter/MF α 1 secretion leader/SMC structural sequence into plasmid pJDB207, using procedures similar to those used with the URA3 plasmids. The structures of the resulting plasmids are shown in Figure 6. The plasmid pJDB207 is described in J. D. Beggs, "Multiple-copy Yeast Vectors," Molecular Genetics in Yeast, Alfred Benzon Symposium 16, 383-95 (1981).

We transformed the three URA-type vectors derived from plasmid pEX-2 (which is described in J. F. Ernst and R. C. Chan, supra) and the three LEU2-type vectors into yeast strain BJ1991. We deposited two of the recombinant yeast strains in the Deutsche Sammlung Von Mikroorganismen (West German Culture Collection). One strain, YE439, is a LEU2-type vector with the ACT promoter/MF α 1 secretion leader/SMC structural sequence. It was deposited on October 30, 1985 and is identified as DSM 3578.

Another strain, YE466, is a LEU2-type vector with the CYC1 promoter/MF α 1 secretion leader/SMC structural sequence. It was deposited on October 30, 1985 and is identified as DSM 3579. Transformants were grown in the "SD" medium described in F. Sherman et al., "Methods in Yeast Genetics," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1981), containing tryptophan and leucine (for URA3-type vectors), or tryptophan and uracil (for LEU2-type vectors) to an optical density of 2 at 600 nm. We used these cultures to inoculate production medium, consisting of SD medium containing 4% casamino acids and tryptophan (inoculum was 10% of final volume of

-26-

production medium). At appropriate times during growth, 0.5 ml of the cultures were pelleted using a microfuge. We redissolved the cell pellets in 1/10 the original culture volume by boiling 5 min in sodium dodecyl sulphate (SDS) sample buffer, which is described in U. K. Laemmli, "Cleavage of the Structural Proteins During the Assembly of the Head of Bacteriophage T4," Nature, 227, pp. 680-85 (1970). We then determined the SMC levels in the redissolved cell pellets and in the culture fluid after dilution, using a radioimmune assay (Nichols Institute Diagnostics, San Juan Capistrano, California).

With all six constructions, less than 10% of the total SMC was cell associated. We purified, SMC present in the culture medium, and analyzed it, finding that the amino terminal and carboxyl terminal amino acids were identical to human SMC and that the biological activity of the secreted SMC was the same as human SMC.

Surprisingly, the weakest promoter used, CYC1, resulted in the highest SMC secretion values shown in Figures 3 and 7 and Table 2. This was unexpected because the strongest promoter would have been expected to give the highest secretion values. Unexpectedly, the transformants carrying the CYC1 constructions grew slowest and showed a pronounced optimum curve of SMC secretion, indicating the occurrence of cell lysis and SMC degradation (see Figure 7).

To determine the reason for the unexpected results, we analyzed SMC plasmid copy number and transcript levels in the transformants described herein after three days in production medium. The results are shown in Table 2. We discovered an inverse relationship between copy number and promoter strength in secretion systems. The secretion system with the weakest promoter (CYC1) proved to have the highest

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copy numbers. The MF α 1 constructs had the lowest copy numbers. The average SMC transcript level proved to be proportional to copy numbers for both URA3 and LEU2 constructs. In turn, with LEU2 constructions, mRNA levels were proportional to secretion levels. With URA3 constructions, no significant increase of SMC secretion levels with increased mRNA was observed. While not wishing to be bound by theory, the URA3 constructs do not appear to have the same effect on the SMC secretion levels because of a high percentage of URA3 plasmid loss.

A high percentage of URA3 transformants actually have few or no URA3 expression plasmids. These unproductive transformants contribute little to overall SMC secretion. Conceivably, a high percentage of URA3 transformants also have high copy numbers and high promoter strength. These transformants would contribute little to SMC secretion because of slow growth and reduced cell viability. This suggested mechanism does not apply to LEU2 vectors because the minimum copy number of the LEU2-type vectors in transformants grown in leucine-free media is about 35 (Erhart and Hollenberg. supra). In addition, since the production medium used contains leucine (but no uracil), elevated copy numbers are not required for growth of LEU2 vector-transformants in this medium. Thus, yeast strains transformed with LEU2 appear more homogeneous than URA3 vectors with respect to copy number and mRNA content.

With the URA3 transcripts, mRNA levels increased

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as promoter strength weakened, even though secretion levels remained fairly constant. This trend appears to reflect the lower copy numbers for URA3 constructs. It appears that, for LEU2 transcripts, a copy number of at least 40 is required to obtain minimum levels of mRNA and SMC secretion.

Table 2 does not give the actual ratio of SMC to actin transcripts, and the numbers are for comparative purposes only.

10 Transformation frequencies for all six plasmid constructions are also shown in Table 2. Approximately similar transformation frequencies were obtained with all the URA3-type vectors. However, dramatic differences were seen for the LEU2-type vectors, the MF α 1 promoter constructions could not be transformed in yeast at all, 15 relatively high frequencies were observed for the ACT promoter construction and lower frequencies of transformation, compared to the control (pJDB207) and the ACT construction, were characteristic of the CYC1 construction.

20 We did obtain one rare LEU2-type vector transformant with the MF α 1 construction; however, this transformant contained a rearranged plasmid that directed the synthesis of low amounts of SMC as shown parenthetically in Table 2. This result suggests that the MF α 1 promoter 25 does not permit a sufficiently high copy number to overcome the effects of the LEU2 defect.

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Thus, secretion rate, cell viability, promoter strength and vector copy number appear to be interrelated.

TABLE 2

<u>Construction</u>	<u>plasmid</u>	<u>promoter</u>	<u>selection</u>	<u>transformants/μg</u>	<u>% loss</u>	<u>copy number</u>	<u>RNA</u>	<u>SMC (mg/l)</u>
1	p336/1	MEal	URA3	225	6	27	10.9	12
2	p364/1	ACT	URA3	319	10	32	26.3	12
3	p446/1	CYC1	URA3	296	12	49	40.2	13
--	p160/1	control	URA3	300	10	45	----	--
4	(pME-SMC) MEal		LEU2	0(1)	(32)	(41)	(8.2)	(8)
5	p482/18	ACT	LEU2	4	0	69	31.9	20
6	p504/5	CYC1	LEU2	2	0	110	54.8	28
--	pJDB-207	control	LEU2	58	0	110	----	--

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EXAMPLE 5Secretion of TNF

We constructed fusions of the MF α 1 secretion leader to the gene encoding human tumor necrosis factor (TNF) as shown in Figure 8 by using the procedure described above for MF α 1/SMC fusions. We then analyzed the effect of promoter replacement on TNF secretion.

We ligated the following three fragments to construct vector pTNF11 (see Figure 8): (1) a 1.2 kb EcoRI-StuI fragment carrying the promoter and the secretion leader of MF α 1 ending in a StuI site (described supra); (2) a 9 kb EcoRI-HindIII fragment of pEX-2 carrying the yeast 2 μ origin of replication and the URA3 gene for selection in yeast (described supra); and (3) a blunt-ended HindIII fragment (0.9 kb) carrying the TNF gene. Isolation procedures for this third fragment are disclosed in D. Pénica et al., "Human Tumor Necrosis Factor: Precursor Structure, Expression and Homology to Lymphotoxin," Nature, 312, pp. 724-29 (1984). We generated the blunt-ended HindIII fragment by using synthetic oligonucleotide linkers to recreate the 5' end of the TNF gene by extension from an AvaI site situated close to the 5' end of the TNF gene (valine is the first amino acid of TNF). The junction of MF α 1 to the TNF gene had the following sequence:

(5')...AAA AGG GTA CGT TCT TCC...(3')

lys arg val arg ser ser

MF α 1 TNF

The construction of a TNF secretion vector based on the ACT promoter, instead of the MF α 1 promoter, is also shown in Figure 8. We ligated the following fragments to construct pACT-TNF-EX: (1) a 1.2 kb PstI-HindIII fragment from pTNF11 carrying most of the MF α 1 secretion leader fused to the TNF

-32-

gene (described supra); (2) a 0.5 kb BamHI-PstI fragment from p355/1, shown in Figure 5, carrying the ACT promoter and part of the MFa1 leader (described supra); and (3) the 9 kb BamHI-HindIII fragment of pEX-2 (described supra).

We transformed yeast strain BJ1991 with pTNF11 or pACT-TNF-EX and selected for Ura⁺ prototrophs. Transformants were grown selectively in liquid SD medium lacking uracil. Shake flasks containing YPD medium, which is disclosed in F. Sherman et al., supra, were inoculated with minimal cultures (10% final volume) and incubated at 30°C on a rotatory shaker. At appropriate times during incubation, we centrifuged 1 ml samples of the yeast culture for 1 min using a microfuge. We then treated the cell pellet with zymolase to generate spheroplasts. We then lysed the spheroplasts using 1% triton X-100 at 20% of the original culture volume, followed by removal of cell debris by a short centrifugation step.

We electrophoresed samples containing TNF on 12.5% SDS-acrylamide gels and transferred the proteins to nitrocellulose by standard methods. We probed the protein blot with rabbit antibodies for human TNF, and we visualized regions of the blot containing TNF/anti-TNF complexes using peroxidase-coupled swine anti-rabbit antibodies. Maximal TNF expression results in the culture fluid and in cell extracts are shown in Table 3. The results indicated that a significant improvement of secretion of TNF was obtained when the MFa1 promoter was replaced by the ACT promoter. Only 10-20% of the total TNF produced was cell associated.

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TABLE 3

MAXIMAL TNF EXPRESSION LEVELS BY YEAST TRANSFORMANTS

	<u>plasmid</u>	<u>cells</u> (mg/litre)	<u>medium</u> (mg/litre)
5	pTNF11	1	5
	pACT-TNF-Ex	1.5	15

EXAMPLE 6Secretion of TPA

The gene coding for human tissue plasminogen activator (TPA) carries a convenient BglII site at a position corresponding to the first amino acid of TPA. D. Pennica et al., "Cloning and Expression of Human Tissue-type Plasminogen Activator cDNA in E. coli," Nature, 301, pp. 214-21 (1983). In order to allow correct fusion to the MFal secretion leader, we introduced a convenient BglII site at the MFal position corresponding to the lys-arg junction. The structure of the MFal/TPA fusion point in the following constructions is as follows:

(5')...AAA AGA TCT TAC...(3')
 lys arg ser tyr
 MFal TPA

We inserted a BglII fragment carrying the MFal promoter and secretion leader into the single BglII site of the previously constructed vector pPAY4 as shown in Figure 9. In this manner, the PHO5 promoter and secretion leader were replaced by the MFal promoter and secretion leader. We further modified the resulting expression vector, pMF-TPA, by replacing the PHO5 and MFal promoters by the ACT promoter, as shown in Figure 9, to obtain expression vector pACT-MF-TPA. The PHO5 promoter present in both TPA constructions is repressed in regular (high-phosphate) production media. Accordingly, the expression results described below are due to the activity of the MFal

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promoter because the tests were carried out in regular (high phosphate) media.

We used each of the expression plasmids, pACT-MF-TPA and pMF-TPA, to transform yeast cells of strain BJ1991, as described above, selecting for Leu⁺ prototrophs. We selectively grew transformants in SD medium. We inoculated shake flasks containing YPD medium with the SD cultures (10% of final volume) and incubated at 30°C. At appropriate times during incubation, we prepared cell extracts as described above in Example 4.

We determined TPA activity in cell fractions by halo formation on fibrinogen-plasminogen-agar plates as described in A. Granelli-Piperno and E. Reich, "A Study of Proteases and Protease-Inhibitor Complexes in Biological Fluids", J. Exp. Med., 148, pp. 223-34 (1978). The results shown in Table 4 indicate that replacement of the ME α 1 promoter by the ACT promoter is advantageous in obtaining secretion of heterologous proteins by yeast using alpha mating factor fusions.

TABLE 4

MAXIMAL TPA EXPRESSION LEVELS BY YEAST TRANSFORMANTS

25	<u>plasmid</u>	<u>cells</u> <u>(μg/litre)</u>	<u>medium</u> <u>(μg/litre)</u>
	pMF-TPA	30	0
	pACT-MF-TPA	100	5

As shown in Table 3, most of the TPA produced was cell associated; only 5% of the total TPA produced appeared in the medium. However, the following evidence suggested that yeast TPA had entered the secretion pathway:

(1) the TPA expressed was biologically active, indicating correct folding. Attempts

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to express TPA in yeast without a secretion signal sequence produced non-active TPA; and

(2) the cell associated TPA was glycosylated. Secretion at least into the lumen of the endoplasmic reitculum is necessary for glycosylation to occur.

It will be apparent to those skilled in the art that various modifications may be made in the invention without departing from its spirit or scope, and our basic construction can be altered to provide other embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments which have been presented as examples.

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WHAT IS CLAIMED IS:

1. A DNA sequence comprising:
 - (a) a promoter of at most intermediate strength; and
 - 5 (b) a DNA secretion signal sequence, beginning with a start codon, heterologous with respect to said promoter and operatively linked to said promoter.
- 10 2. The DNA sequence of claim 1, wherein said start codon is ATG.
3. The DNA sequence of claim 1 or 2, wherein said promoter is selected from the group consisting of: the ACT, CYC1, CYC7, trp1, URA3, LEU2, trp and lac promoters.
- 15 4. The DNA sequence of any one of the preceding claims, wherein said DNA secretion signal sequence is the MF α 1 secretion signal sequence.
5. A DNA sequence according to any one of the preceding claims further comprising a DNA
20 sequence coding for a desired protein operatively linked to said DNA secretion signal sequence.
6. The DNA sequence of claim 5, wherein said desired protein is capable of being secreted by a microorganism or cell.
- 25 7. The DNA sequence of claim 5 or 6, wherein said desired protein is selected from the group consisting of serum proteins, analgesic polypeptides, β -endorphin, somatostatin, SMC, insulin, human growth hormone, bovine growth hormone,
30 luteinizing hormone, ACTH, pancreatic polypeptide

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preproteins, TPA, TNF, preproinsulin, proinsulin,
the A chain of insulin and the B chain of insulin.

8. A multicopy expression vector
comprising a DNA sequence according to any one of
5 claims 1-7.

9. The multicopy expression vector of
claim 8, wherein said multicopy expression vector is
selected from the group consisting of plasmids and
multicopy phages.

10 10. A host transformed by the DNA sequence
of any one of claims 1-7.

11. A host transformed by the multicopy
expression vector of any one of claims 8-9.

12. The host of any one of claims 10-11,
15 wherein said multicopy expression vector is selected
from the group consisting of: ARS-type plasmids,
2 μ -type plasmids, ColE1 type plasmids, pBR322, RP4
plasmid, M13 phage and lambda phage.

13. The host of any one of claims 10-12,
20 wherein said promoter does not materially reduce the
copy number of said multicopy expression vector in
said host.

14. The host of any one of claims 10-13,
wherein the expression control sequence of said
25 promoter produces less than 0.3 percent of the total
mRNA within said host.

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15. The host of claim 14, wherein at most 0.15 percent of the total mRNA within said host is the product of said promoter.

16. The host of any of claims 10-15, wherein
5 said host is selected from the group consisting of: bacteria, fungi, yeasts, animal cells, plant cells in tissue culture or human tissue cells.

17. The host of any one of claims 10-16,
10 wherein said host is selected from the group consisting of: E.coli, Bacillus, Streptomyces, Pseudomonas, S. cerevisiae, CHO cells, mouse cells, swine cells, bovine cells, fowl cells or fish cells.

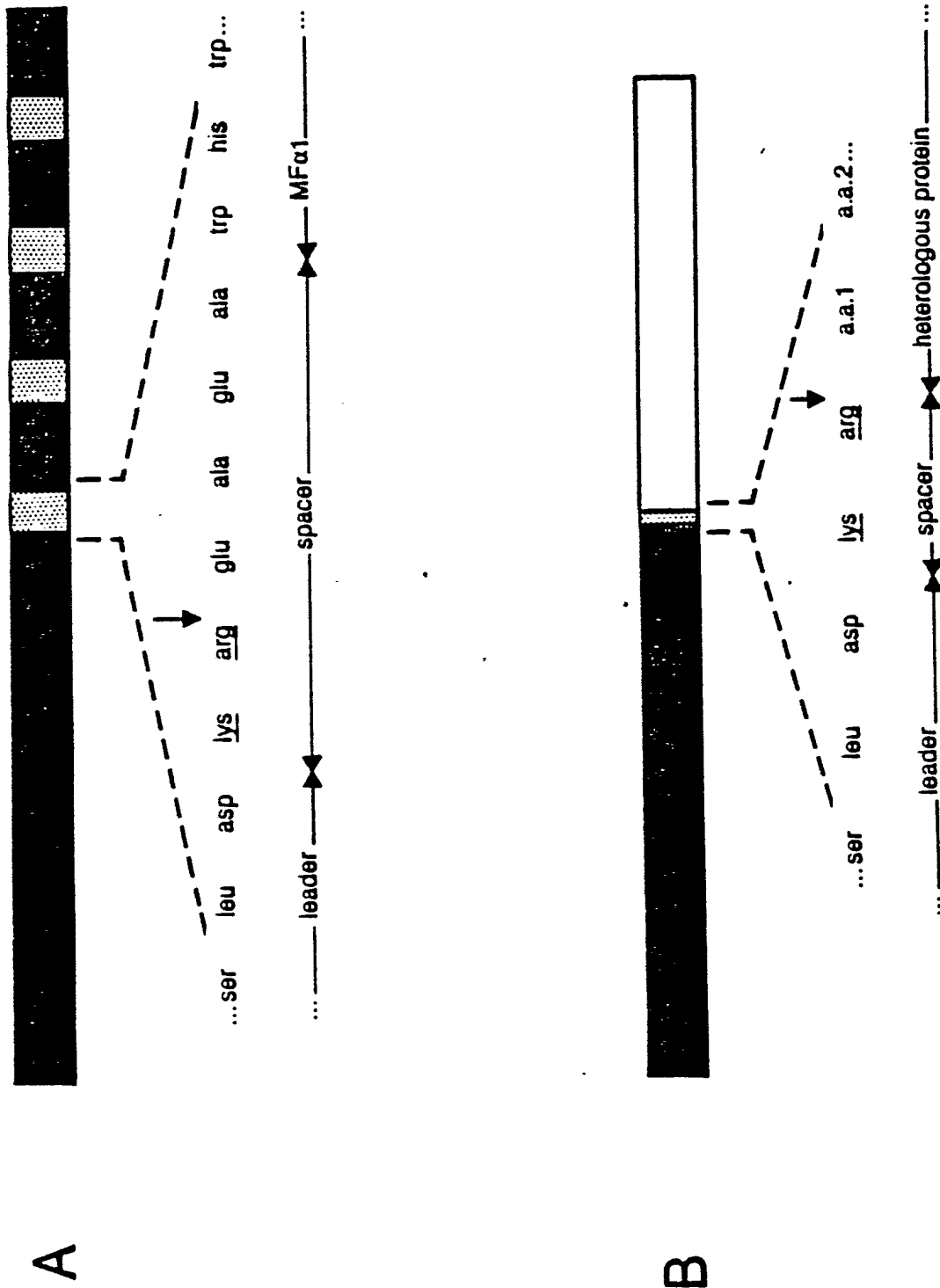
18. The host of any one of claims 10-17, wherein said host is yeast.

15 19. The host of claim 18, wherein said yeast is S. cerevisiae.

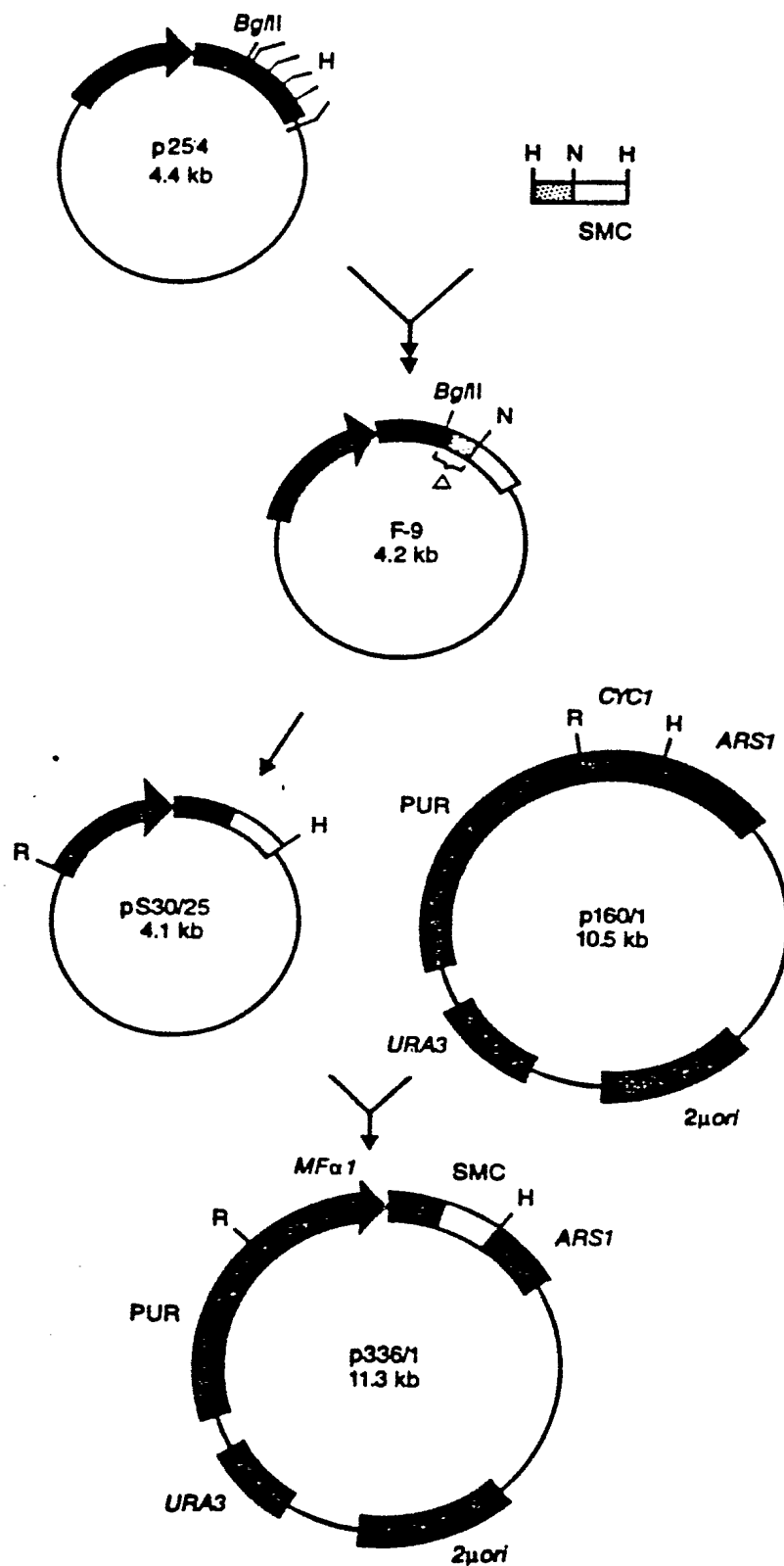
20. A method for producing a desired protein, comprising culturing the host of any one of claims 10-19.

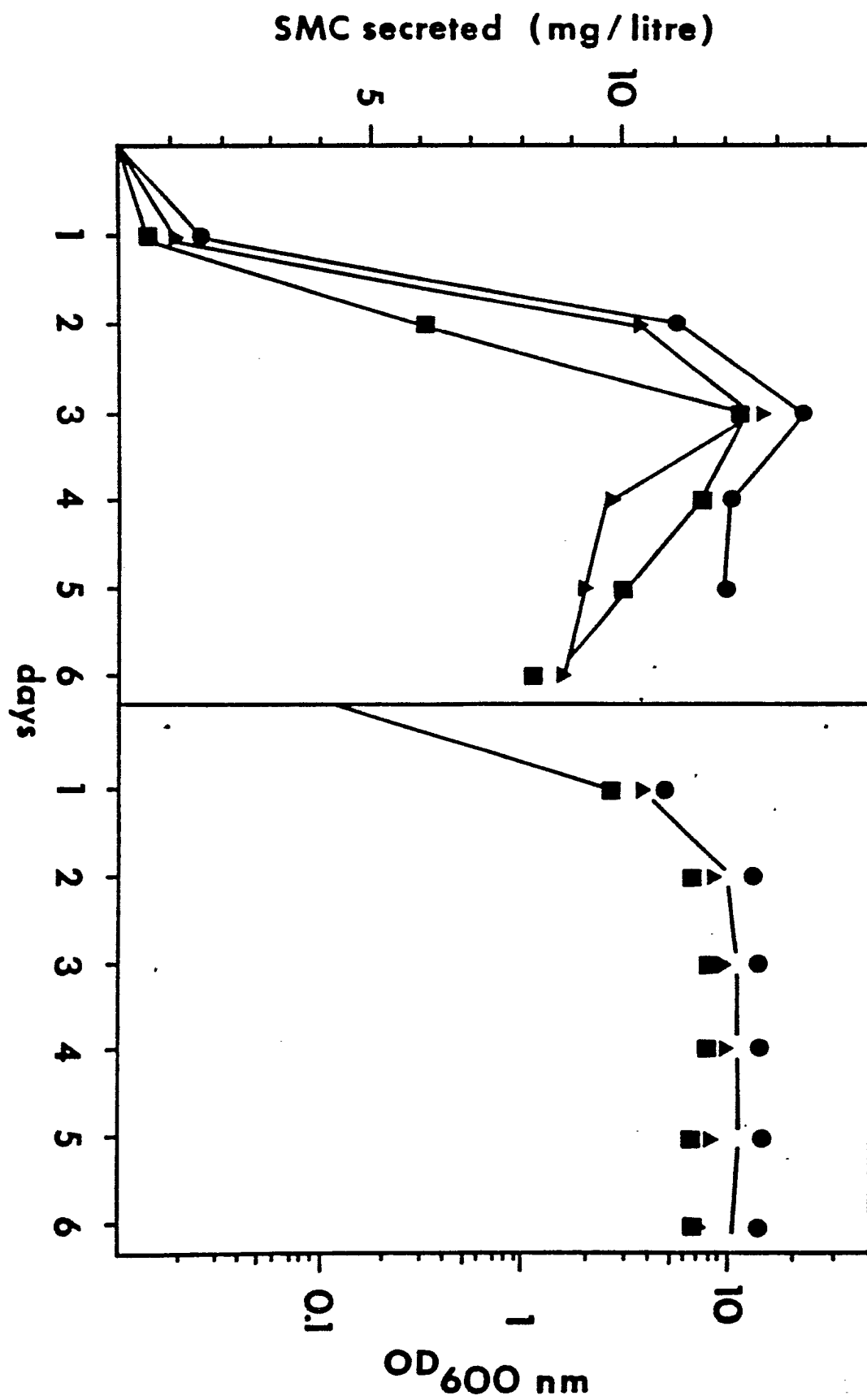
1/a

Figure 1

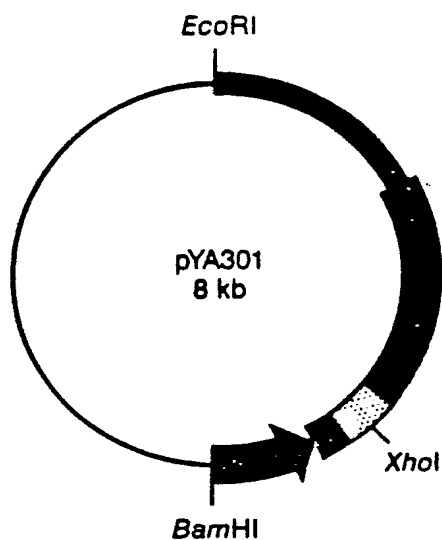


2/a





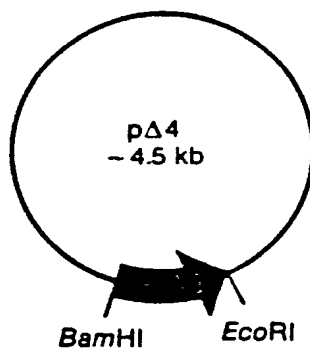
A



1. digest with *XhoI*
2. treat with Bal31
3. digest with *EcoRI*
4. fill-in with dNTPs
5. religate

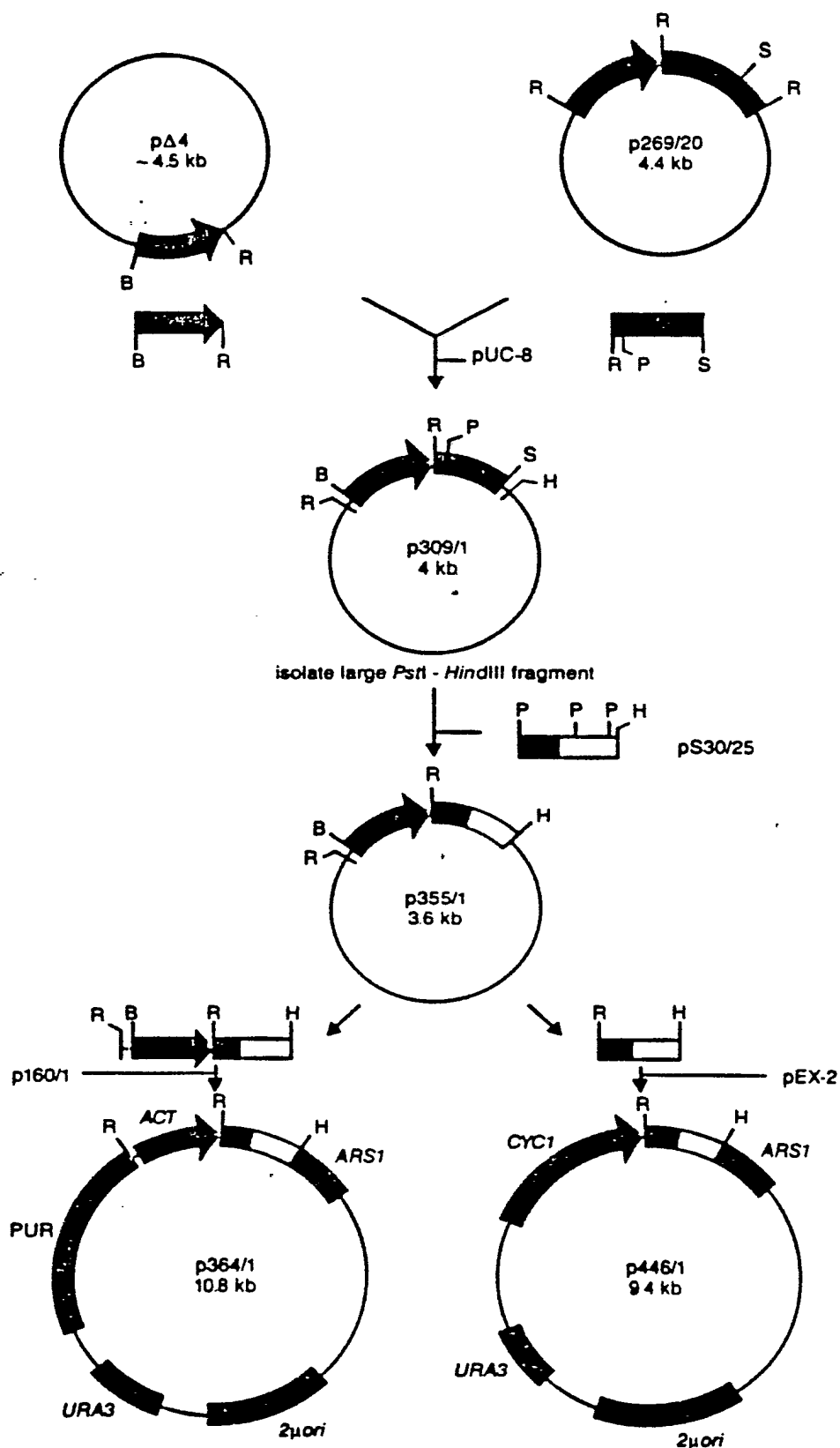


B



	-20	-10	+1
Actin	GATCGAAAATTTACTGAATTAACAATGGATTCTGG		
pEX-5	<u>GAATTC</u>		
pEX-7	GATCGAAAATTTACTGAATTC		
pEX-8	GATCGAAAATTTACTGAATTAACAATGGATTCTGAATTC		

Figure 5



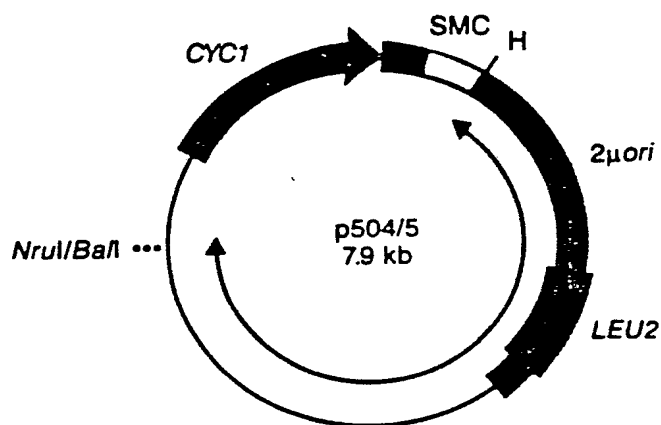
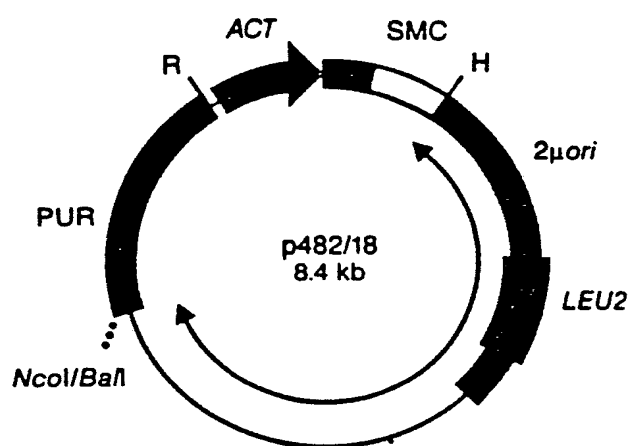
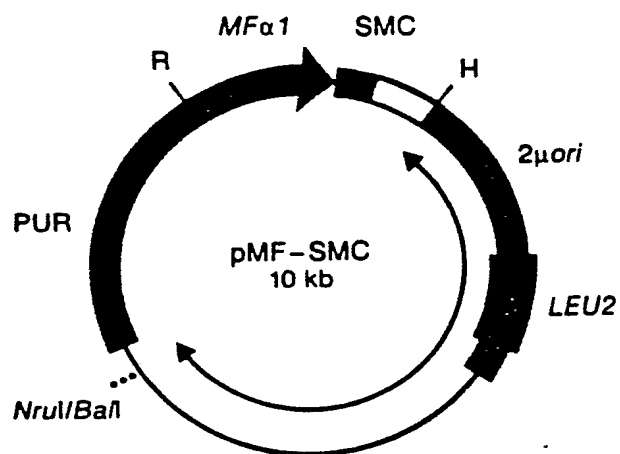


Figure 7

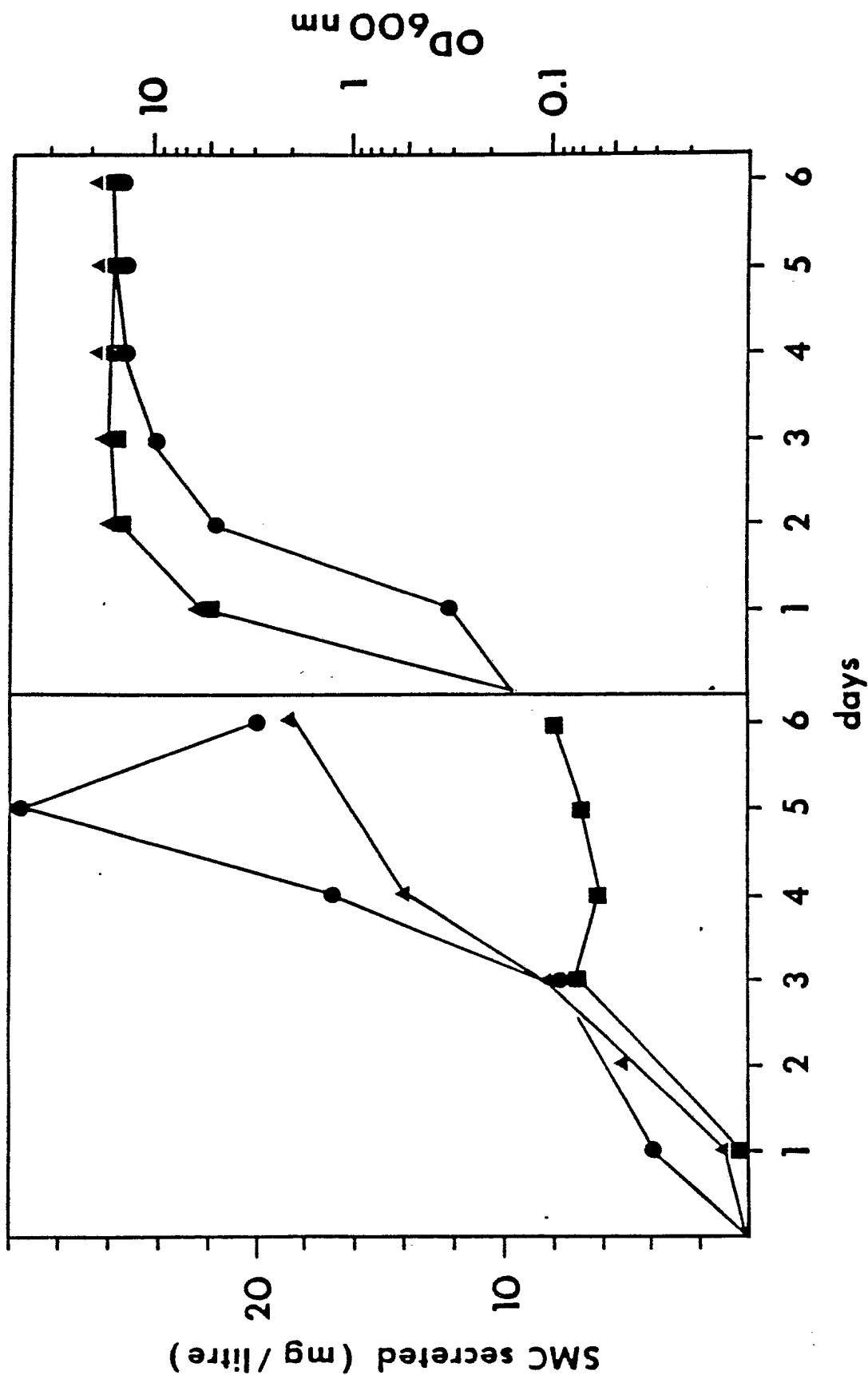
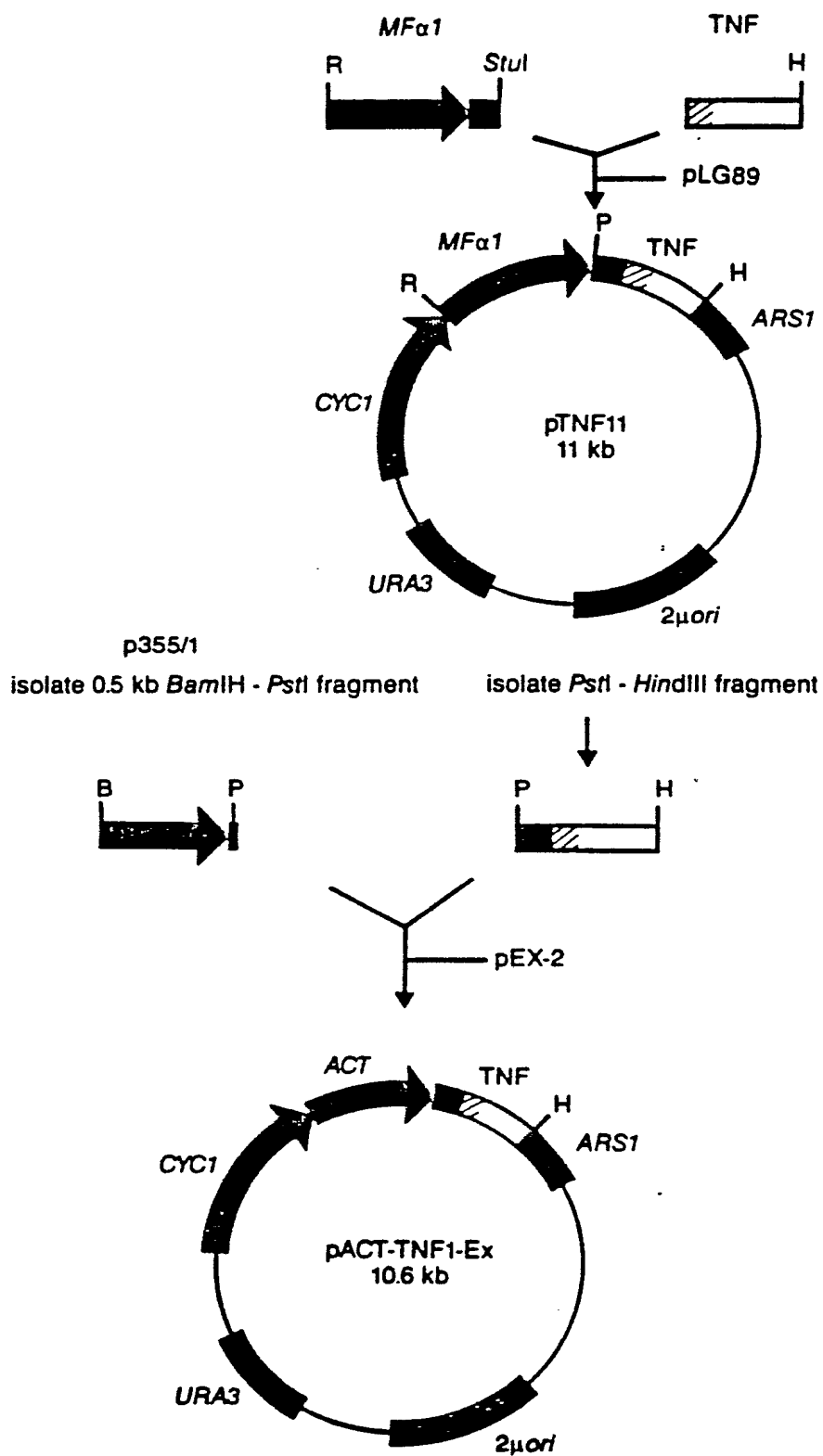
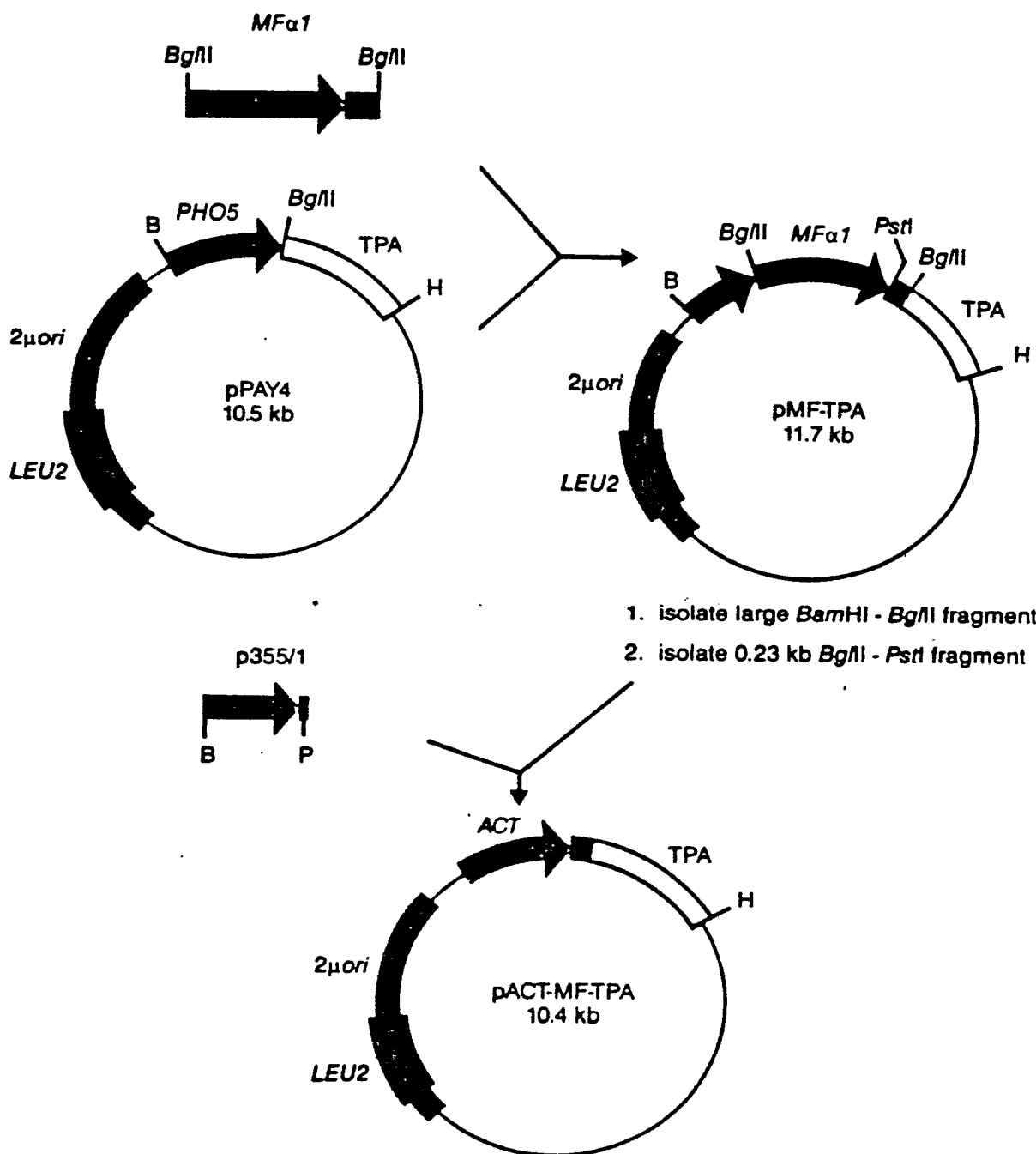


Figure 8





INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 86/00675

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 15/00; C 12 N 1/18; C 12 N 1/20; C 12 N 5/00 C 12 P 21/00																	
II. FIELDS SEARCHED <div style="text-align: right; margin-right: 100px;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC⁴</td> <td style="padding: 5px;">C 12 N; C 12 P</td> </tr> </table> <div style="text-align: center; margin-top: 10px; font-size: small;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ </div>			Classification System	Classification Symbols	IPC ⁴	C 12 N; C 12 P											
Classification System	Classification Symbols																
IPC ⁴	C 12 N; C 12 P																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; text-align: left; border-bottom: 1px solid black;">Category ⁹</th> <th style="text-align: left; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="text-align: left; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Nucleic Acids Research, volume 11, no. 8, 1983, M.J. Dobson et al.: "Expression in saccharomyces cerevisiae of human interferon-alpha directed by the TRP1 5' region", pages 2287-2302 see page 2295, paragraph 2; page 2296 --</td> <td style="vertical-align: top; padding: 5px;">1-3, 5, 8-20</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Gene, volume 35, 1985, M.A. Peters et al.: "Expression of a biologically active analogue of somatomedin-C/insulin-like-growth factor I", pages 83-89 see the whole document --</td> <td style="vertical-align: top; padding: 5px;">1, 3, 5, 7-13, 16, 17, 20</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Nature, volume 287, 2 October 1980, D.V. Goeddel et al.: "Human leukocyte interferon produced by E. coli is biologically active", pages 411-416 see page 414, column 2 --</td> <td style="vertical-align: top; padding: 5px;">1-3, 5, 8-13, 16, 17, 20</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X, P</td> <td style="padding: 5px;">EP, A, 0200590 (GENETICA) 5 November 1986 see page 16, lines 22-29 --</td> <td style="vertical-align: top; padding: 5px;">1-3, 5, 8-13, 16, 17, 20</td> </tr> </table> <div style="font-size: x-small; margin-top: 10px;"> <p>⁹ Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p style="margin-left: 150px;">----- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Nucleic Acids Research, volume 11, no. 8, 1983, M.J. Dobson et al.: "Expression in saccharomyces cerevisiae of human interferon-alpha directed by the TRP1 5' region", pages 2287-2302 see page 2295, paragraph 2; page 2296 --	1-3, 5, 8-20	X	Gene, volume 35, 1985, M.A. Peters et al.: "Expression of a biologically active analogue of somatomedin-C/insulin-like-growth factor I", pages 83-89 see the whole document --	1, 3, 5, 7-13, 16, 17, 20	X	Nature, volume 287, 2 October 1980, D.V. Goeddel et al.: "Human leukocyte interferon produced by E. coli is biologically active", pages 411-416 see page 414, column 2 --	1-3, 5, 8-13, 16, 17, 20	X, P	EP, A, 0200590 (GENETICA) 5 November 1986 see page 16, lines 22-29 --	1-3, 5, 8-13, 16, 17, 20
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IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 25th February 1987 </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report - 2 APR 1987 </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer M. VAN NIEUW </td> </tr> </table>			Date of the Actual Completion of the International Search 25th February 1987	Date of Mailing of this International Search Report - 2 APR 1987	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer M. VAN NIEUW											
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO.

PCT/EP 86/00675 (SA 15262)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 10/03/87

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0200590	05/11/86	FR-A- 2579224	26/09/86
		JP-A- 61275223	05/12/86

For more details about this annex :

see Official Journal of the European Patent Office, No. 12/82